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In re application of:

MARTINS-GREEN, Manuela et al.

Application No.: 09/811,162

Filed: March 16, 2001

For: CHEMOKINES AND METHODS FOR INDUCING THE DIFFERENTIATION OF FIBROBLASTS TO MYOFIBROBLASTS

Examiner: Regina M. DeBerry

Art Unit: 1647

DECLARATION UNDER 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

DECLARATION OF DR. MANUELA MARTINS-GREEN

I, Dr. Manuela Martins-Green, am an Associate Professor of Cell Biology at the University of California, Riverside, and a co-inventor of the above-referenced application. This Declaration provides evidence that IL-8 stimulates the differentiation of fibroblasts to myofibroblasts. Figures showing immunofluorescence studies and immunoblots studies using IL-8 or IL-8 fragments are provided, which were carried out essentially as described for cCAF in the Example (Section B, part 6 and 7) of the above-referenced application. Specifically, immunofluorescence studies were carried out on cultured fibroblasts treated with IL-8, which were then stained for α-smooth muscle actin (α-SMA), a marker for myofibroblast differentiation. Immunoblots were also carried out on cultured fibroblasts treated with IL-8 or IL-8 fragments, which were then stained for α-SMA protein production.

Immunofluorescence studies show that fibroblasts treated with interleukin-8 (IL-8) are positive for α-smooth muscle actin (α-SMA), a marker for myofibroblast differentiation.

Cultured human fetal lung fibroblasts (HFL-1) were treated for three days with melanoma growth stimulatory activity (MGSA) (B), or with IL-8 (C), or were untreated (control) (A) and stained with an anti-α-SMA antibody (Sigma). α-SMA is a marker for myofibroblast differentiation. The cultures were treated with 1000 ng/ml of MGSA or IL-8. The media containing the chemokine, MGSA or IL-8, was replaced approximately every 24 hours. After 3 days, the cells were rinsed with phosphate buffer saline (PBS) and the cells were fixed with 4% paraformaldehyde. Following fixation, the cells were incubated with PBS containing 0.1 M glycine for 10 minutes and blocked for 30 minutes with 10 % goat serum and 0.1% Triton X-100 in PBS. The cells were then incubated with mouse anti-α-SMA (1:200) in 1 % bovine serum albumin (BSA)/PBS for 1 hour at room temperature, and washed three times with 0.1% BSA/PBS for 10 minutes each. The cells were incubated in goat anti-mouse Alexa (1:100) in 1% BSA/PBS for 1 hour at room temperature, washed three time for 10 minutes with 0.1% BSA in PBS and mounted with Vectashield. The results of these studies are shown in attached Figure 1, Panels A, B, and C. Figure 1, Panels A, B, and C show cells stained for α-SMA after the various treatments described above. Panel A shows untreated (control) cells, Panel B shows cells treated with MGSA and Panel C shows cells treated with IL-8. The cells treated with MGSA (B) and IL-8(C) have more cells producing α-SMA and are more intensely stained for α-SMA than control cells.

Immunoblots studies show that fibroblasts treated with IL-8 are positive for α-SMA, a marker for myofibroblast differentiation.

Cultured HFL-1 cells were treated with either 500 ng or 1000 ng of forms of IL-8 or were untreated (control) and then analyzed by immunoblot for production of α-SMA. The forms of IL-8 used in these studies were a 72 amino acid (72aa) form and 77 amino acid (77aa) form. These forms are indicated in the above referenced application as IL-8-72 (SEQ ID NO: 5) and IL-8-77 (SEQ ID NO: 4) at page 26. The cells were incubated for three days as described above. After the three days of treatment, protein extracts were isolated from the control and IL-8 treated cells in 150 mM RIPA buffer containing protease inhibitors. A DC protein assay kit (Bio-Rad) was used to determine protein concentrations and equal amounts of protein were loaded in each well. Samples were run on a 7.5% separating SDS-PAGE Doucet gels. The protein was transferred in the gels to nitrocellulose using a wet-transfer apparatus (Bio-Rad) at 100 volts for 45 minutes. The nitrocellulose membranes were blocked for 1 hour in 5% milk in TTBS and then incubated overnight

at 4°C with mouse anti-α-SMA (1:1500) in 1% milk in TTBS. The nitrocellulose membranes were washed three times for 10 minutes each with TTBS, incubated with anti-mouse HRP at 1:10,000 in 1% milk for 1 hour and washed as above. The bands were visualized using ECL. As seen in attached Figure 1, panel E, the results show that treatment with both the 72 amino acid (72aa) and 77 amino acid (77aa) forms of IL-8 stimulated α-SMA expression. The immunoblot is shown in the upper part of the figure with the relative density of the spot indicated below. Lane 1 shows the control fibroblasts, while lanes 2-5 show the α-SMA detected when HFL-1 are treated with various concentration of IL-8 and various forms of IL-8 as indicated in the figure. All lanes contain equal amounts of total protein, as measured by the DC protein assay. In conclusion, when human fibroblasts are treated with IL-8, they express more α-SMA protein than untreated cells.

Immunoblots studies show that fibroblasts treated with N-terminal fragments of IL-8 are positive for α-SMA, a marker for myofibroblast differentiation.

Cultured HFL-1 cells were treated with 1000ng/ml of either IL-81 polypeptide or IL-8s polypeptide or untreated (control) and analyzed by immunoblot for production of α-SMA. IL-81 polypeptide has the amino acid sequence AVLPRSAKELR and IL-8s polypeptide has the amino acid sequence SAKELR. These polypeptides are identified in the Application as IL-8-77 (SEQ ID NO:9) and IL-8-72 (SEQ ID NO: 8) at page 27, respectively. Immunoblotting was carried out as described above. As seen in attached Figure 2B, the results show that treatment with the N-terminal fragments of IL-8 (IL-8l or IL-8s), stimulated α-SMA expression. The immunoblot is on the top part of the figure with the relative densities of the spots on the bottom part of the figure. Lane 1 shows a control lane, lane 2 shows treatment of HFL-1 cells with MGSA and lanes 3 and lane 4 show treatment with IL-8 polypeptides, IL-8l and IL-8s. All lanes contain equal amounts of total protein, as measured by the DC protein assay. This figure demonstrates that an N-terminal portion of IL-8 contains the active domain that stimulates myofibroblast differentiation.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge what willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's Signature:

Manuela Martins-Green

Manuela Martins-Green, Ph.D.

10/31/02

Date